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Rapid Communication

p53 Expression in Colorectal Tumors

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The expression of the nuclear phosphoprotein p53 was studied immunohistochemically in a series of 150 benign and malignant colorectal tumors. Using monoclonal antibody PAb1801, tumors divided unequivocally into two groups on the basis of immunohistochemistry. Forty of the carcinomas (46.5%) showed positive staining but only 4 of the adenomas (8.7%) were positive ($P < 0.001$). The few positive adenomas always showed moderate or severe dysplasia. Metaplastic polyps ($n = 9$) and small familial adenomatous polyposis-related adenomas ($n = 9$) were uniformly negative. Carcinomas with p53 expression did not differ from those without in terms of site, differentiation or the prognostic indicators of Dukes' stage, DNA ploidy, or tumor histology. The improved morphologic resolution available in periodate lysine paraformaldehyde dichromate (PLPD)-fixed, paraffin-embedded tissue permitted several conclusions to be made: p53 is confined to neoplastic nuclei; staining in positive tumors is heterogeneous and often more marked at the infiltrative margins; and staining intensity is dramatically reduced in mitotic cells. It is concluded that expression of immunohistochemically detectable p53 (probably representing mutated forms of the protein) occurs in some adenomas around the time of transition to carcinoma. Therefore there is an association with the appearance of infiltrative behavior but not with degree of tumor progression (including metastasis) at the time of resection. (Am J Pathol 1991, 138:807–813)

Overexpression of p53 has been demonstrated immunohistochemically in a number of common human malignancies, including carcinomas of colon, breast, and lung.[1–3] In the case of breast carcinoma, this immunoreactivity has been shown to correlate with a number of clinical and histopathologic indicators of poor prognosis[2] and may become useful in the future in determining therapeutic strategies.

The 53-kd nuclear phosphoprotein p53 is found in all mammalian cells so far investigated as well as in xenopus.[4] It is usually expressed at low levels and has a short half-life (only 6 minutes in the spleen).[5] Its normal function remains obscure but it has been implicated in control of the cell cycle because levels of p53 mRNA and protein increase in late G1 before the onset of DNA synthesis[6] and cells are prevented from entering S phase by microinjection of p53 antibodies or by the presence of anti-sense p53 RNA.[7,8]

p53 forms stable complexes with the products of transforming genes of DNA viruses (SV40 large T antigen[9,10] and adenovirus E1b protein[11]) and can be oncogenically activated by mutations in certain regions of the gene.[12] Under these circumstances, p53 becomes much more stable,[13] loses the capacity to bind viral oncogenes,[14] immortalizes rodent fibroblasts,[15–17] and cooperates with *ras* in their transformation.[18] The nonmutated, wild-type (WT) p53 gene, however, behaves as a tumor suppressor and can reverse the transformed phenotype.[19]

Interest in p53 in colorectal cancer was stimulated by the finding that the region on chromosome 17p, which is consistently deleted in the majority of such tumors (thus indicating the site of a putative tumor suppressor gene), contains the gene for p53.[20] In cases in which one copy of p53 is deleted, the remaining copy is frequently mutated, but mutations also occur in tumors that retain a normal p53 allele.[21]

Previous immunohistochemical studies of p53 have been restricted to frozen sections,[1,2] because the epitope is destroyed by formaldehyde fixation. Here, by the use of the fixative PLPD,[22] we exploited the superior morphologic resolution of immune reaction product pos-

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sible in paraffin sections. We describe p53 expression in a series of 86 colorectal carcinomas and 62 polyps, including 9 from patients with familial adenomatous polyposis, and correlate the staining pattern with well-recognized indicators of clinical outcome.

Materials and Methods

Tissue

Ninety-two consecutive colectomy specimens were received onto ice in the operating theater, opened, and rinsed in ice-cold phosphate-buffered saline/ethylenediamine tetraacetic acid (EDTA) (20 mmol/l [millimolar] sodium phosphate buffer pH 7.4, 145 mmol/l sodium chloride, 0.5 mmol/l EDTA) within 30 minutes. Samples of tumor and adjacent normal mucosa for immunohistochemistry were placed immediately in periodate lysine paraformaldehyde dichromate (PLPD) fixative[22] (1% paraformaldehyde, 75 mmol/l lysine, 37.5 mmol/l sodium periodate, 2.5% potassium dichromate in 18.75 mmol/l sodium phosphate buffer pH 7.4) fixed for 24 hours at 4°C, washed extensively, and processed into paraffin blocks. Further samples of tumor and distant normal mucosa were snap frozen in liquid nitrogen and then stored at −70°C for flow cytometry or cryostat sections. The rest of the specimen was fixed in formalin and processed routinely for hematoxylin and eosin staining and histopathologic assessment. For the purposes of numerical analysis, tumors were defined as right sided if they lay between cecum and splenic flexure or left sided if they were more distal.

Histopathologic Grading

Carcinomas were reviewed and graded by a single pathologist (J Piris) using criteria recommended by the United Kingdom Coordinating Committee on Cancer Research for Dukes' stage, histologic type, degree of differentiation, and nature of invasive margin.[23] Adenomas were similarly reviewed and graded by a single pathologist (J. O'Grady) for morphologic type and degree of dysplasia.[24]

Flow Cytometry

Three or more samples from separate sites in each carcinoma were prepared for assessment of nuclear DNA content by flow cytometry using the method of Vindelov.[25] The tissue was disaggregated under a citrate buffer and treated with detergent (0.1% NP-40) and trypsin (30 μg/ml) followed by RNAase A (0.1 mg/ml). The resulting nuclear suspension was stained with propidium iodide at a final concentration of 2 mg/ml. Avian erythrocytes were added as an internal standard and the samples analysed on a Coulter EPICS CS flow cytometer (Coulter, Hialeah, FL) at an excitation wavelength of 488 nm. At least 10,000 nuclei were analyzed in each sample and cases were scored as aneuploid if any of the three samples showed a G_0/G_1 peak distinct from the normal diploid peak (as assessed from the internal standard). Coefficients of variation of the diploid peak were typically less than 3.5.

Immunohistochemistry

Three-micrometer sections of PLPD-fixed, paraffin-embedded tissue were stained using the human p53-specific mouse monoclonal antibody PAb1801[26] (p53-Ab2 Oncogene Science Inc., Manhasset, NY). Sections were rehydrated in graded alcohols incubated in normal rabbit serum diluted 1:5 in TRIS-buffered saline (TBS) (145 mmol/l NaCl, 20 mmol/l TRIS pH 7.6), and exposed to primary antibody at a dilution of 1:100 for 1 hour. Bound antibody was detected using biotinylated rabbit antibody to mouse immunoglobulin (Dakopatts No. E354, Glostrup, Denmark) and avidin-biotin complex linked to horseradish peroxidase (Dakopatts No. K335). Copious washing in TBS between each step was essential. Visualization was with diaminobenzidine (1 mg/ml) in the presence of 0.03% hydrogen peroxide. Endogenous peroxidase was not inhibited. Cases known to stain positively were included in each run, receiving either primary antibody or simply dilution buffer, to monitor consistency and act as controls. Sections received a light hematoxylin counterstain and were dehydrated in graded alcohols and xylene before mounting. The specificity of PAb1801 for human p53 under the conditions used was confirmed by inclusion in some staining runs of PLPD-fixed SV80 cells (an SV40-transformed, human fibroblast cell line known to express high levels of p53).[27] In addition, 4-μm cryostat sections from a number of carcinomas in the series were fixed in acetone for 15 minutes then exposed to PAb1801 at a dilution of 1:100 (53 cases) or undiluted culture supernatant from the hybridoma secreting antibody PAb240 (39 cases). PAb240 binds to a different p53 epitope from PAb1801, revealed by the conformational changes induced by mutation.[28] Bound antibody was detected using rabbit antibodies to mouse immunoglobulins conjugated with horseradish peroxidase (Dakopatts No. P260) at a dilution of 1:20 and visualized in an identical fashion to that used for PLPD-fixed sections. The distribution of PAb1801 staining within cells and within the tissue was identical in PLPD-fixed and frozen section. No

discrepancy in the assignment of PAb1801 positivity between frozen and paraffin sections was found.

Immunoblots

Total protein was extracted from tumors, normal colonic mucosa, and SV80 cells. Equal amounts of total protein (approximately 50 μg) were separated by electrophoresis through a 10% polyacrylamide gel containing 0.1% sodium dodecyl sulfate along with molecular-weight standards (MW-SDS-70L Sigma Chemical Co., St. Louis, MO) and transferred to nitrocellulose (Hybond-C, Amersham, Arlington Heights, IL) by standard methods.[28] Nonspecific binding was inhibited by overnight incubation in TBS, 10% milk protein, 0.1% Tween-20, then probed with PAb1801 (1:200) and detected using a streptavidin, biotin, alkaline-phosphatase system (Amersham, RPN 22) in accordance with the manufacturers' recommendations.

Results

Reaction product, marking the binding of PAb1801, was confined to the nuclei of neoplastic cells (Figure 1a). Normal colonic mucosa never stained in cytoplasm or nucleus. Rarely pale staining was observed in smooth muscle cells (particularly of blood vessels) at high antibody concentrations but not at the working dilution. Staining

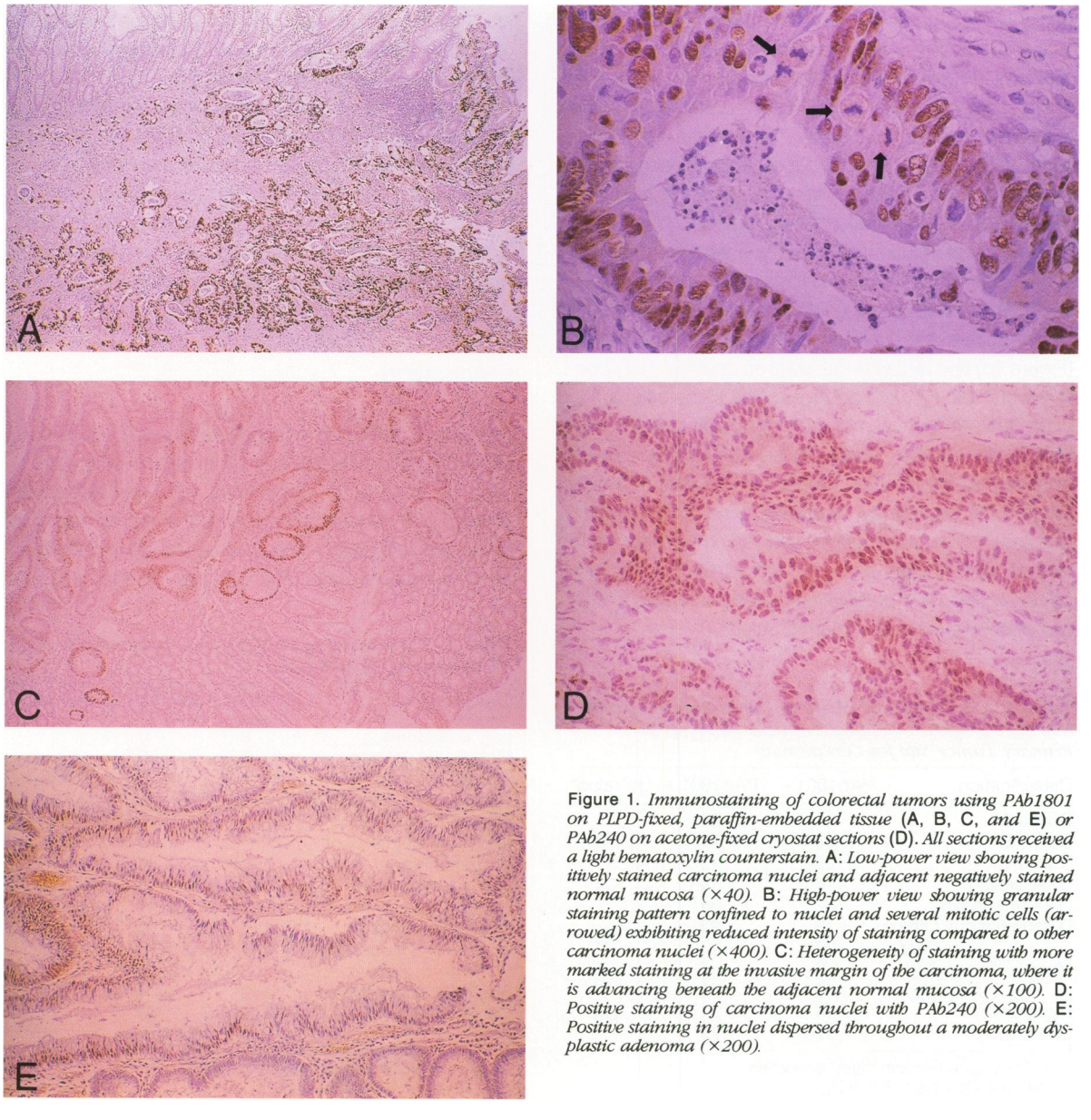

Figure 1. *Immunostaining of colorectal tumors using PAb1801 on PLPD-fixed, paraffin-embedded tissue* (A, B, C, *and* E) *or PAb240 on acetone-fixed cryostat sections* (D). *All sections received a light hematoxylin counterstain.* A: *Low-power view showing positively stained carcinoma nuclei and adjacent negatively stained normal mucosa (×40).* B: *High-power view showing granular staining pattern confined to nuclei and several mitotic cells (arrowed) exhibiting reduced intensity of staining compared to other carcinoma nuclei (×400).* C: *Heterogeneity of staining with more marked staining at the invasive margin of the carcinoma, where it is advancing beneath the adjacent normal mucosa (×100).* D: *Positive staining of carcinoma nuclei with PAb240 (×200).* E: *Positive staining in nuclei dispersed throughout a moderately dysplastic adenoma (×200).*

within the tumor nuclei was granular or reticular in nature (Figure 1b) sometimes with obvious nucleolar sparing. During mitosis, reaction product was reduced dramatically (Figure 1b), whereas interphase tumor cells never showed cytoplasmic staining.

The tumors divided unequivocally into two groups on the basis of PAb1801 immunohistochemistry. Tumors were scored as positive when staining was visible in any nuclei within the tissue section. In such cases, positive nuclear staining of identical incidence and distribution was found even when the concentration of primary antibody was reduced to 1:400 or increased to 1:10. Similarly cases scored negative remained without staining in any cells even at concentrations as high as 1:10.

In positive tumors the pattern of immunostaining was not uniform. In a small number of tumors almost all the nuclei stained positively, but the more common pattern was an admixture of tumor acini that showed no staining with those in which most nuclei were stained. The greatest proportion of positive cells was found in acini at the invasive margins of carcinomas. Sometimes the distinction was obvious within a single gland, with strong staining on the outer side and weak or none on the inner side (Figure 1c). All these patterns were sustained over a 40-fold range of dilution of primary antibody (data not shown). Immunostaining of cryostat sections from a subset of 39 carcinomas using PAb240 demonstrated a very similar pattern and overall incidence of antibody binding (Figure 1d) and all cases that were scored PAb1801 positive were also PAb240 positive by the same criteria.

In total, 86 carcinomas from 86 patients were analyzed with PAb1801 and of these 40 (46.5%) showed positively stained nuclei. Prognostic indicators (Duke's stage, DNA aneuploidy, degree of tumor differentiation, and nature of invasive margin) showed no significant correlation with PAb1801 staining (Table 1). Similarly the proportion of PAb1801-positive tumors was unrelated to their site within the colon or rectum (Table 1).

Table 1. *Correlation of PAb1801 Immunostaining with Histopathological Classification, DNA Ploidy, and Primary Tumor Site for Carcinomas*

Classification	PAb1801 −	PAb1801 +	p Value*
Dukes A	1	4	
Dukes B	23	21	NS
Dukes C	22	15	
DNA diploid	14	6	NS
DNA aneuploid	32	34	
Differentiation: Other	19	23	NS
Poor	27	17	
Margin: Expanding	6	5	NS
Infiltrating	40	35	
Right colon	14	11	NS
Left colon	32	29	

* Chi-square test with Yates' correction.
NS, not significant ($P > 0.05$).

Fifty-five sporadic polyps from 30 patients were studied with PAb1801 comprising 9 metaplastic polyps and 46 adenomas. The metaplastic polyps were all negative. Of the 46 adenomas, 4 (8.7%) were positive (Table 2). The positively staining nuclei were dispersed throughout the adenoma in one case (Figure 1e) and in the remaining three were restricted to only a few glands. All four showed either moderate or severe dysplasia, but there is no statistically significant correlation within our series with other morphologic parameters or tumor size (Table 2). In one large villous adenoma that contained foci of infiltrative carcinoma, however, p53 staining was absent in the adenoma but present in the infiltrative glands only. In addition, PLPD-fixed material was available from nine tubular adenomas, all less than 4 mm in diameter from two patients undergoing colectomy for FAP; all were negative for PAb1801 immunostaining. The difference in incidence of positive staining in adenomas as compared with carcinomas is very significant (Table 2).

Immunoblots of total protein extracted from tissue samples (Figure 2) demonstrated that PAb1801 recognizes a single 53-kd protein that could be extracted from those tumors that were immunohistochemically positive but not from negative tumors or normal colonic mucosa. This detection of p53 in immunoblots correlated exactly with detection in frozen and paraffin sections.

Discussion

The data demonstrate a strong relationship between p53 expression and malignant transformation in colorectal tumors. In contrast to carcinomas, very few adenomas showed p53 expression and those that did all belonged to the more dysplastic categories in which the risk of transition to invasive carcinoma is higher. In one case of a

Table 2. *Correlation of PAb1801 Immunostaining with Histopathology of Adenomas*

Classification	PAb1801 −	PAb1801 +	p Value*
FAP adenomas	9	0	
Metaplastic polyps	9	0	
Tubular adenomas	1	2	NS
Tubulovillous adenomas	11	2	
Villous adenomas	12	0	
Maximum diameter of adenoma†			
≥10mm	29	3	NS
<10mm	13	1	
Dysplasia† Mild	5	0	
Moderate	34	2	NS
Severe	3	2	
Sporadic adenomas	42	4	$P < 0.001$
Carcinomas	46	40	

* Chi-square test with Yates' correction.
NS, not significant ($P > 0.05$).
† Sporadic adenomas only.

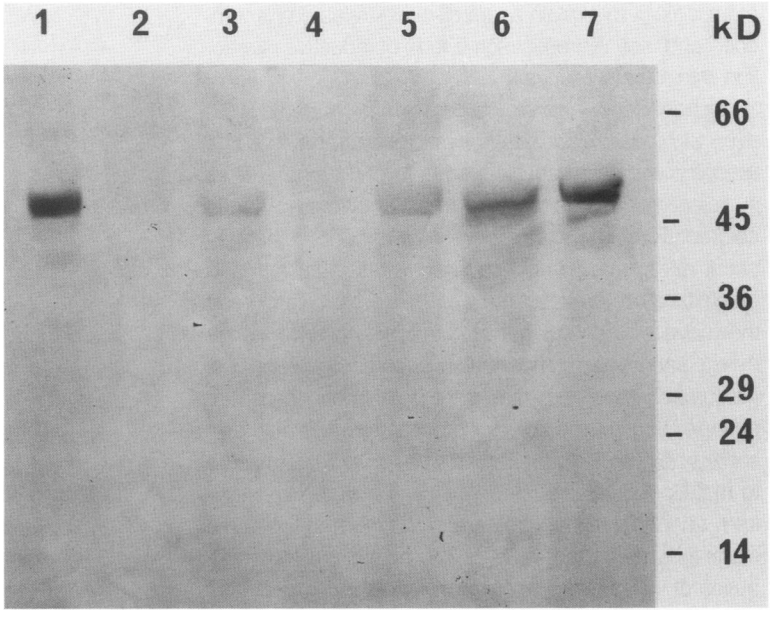

Figure 2. *Immunoblot of total protein stained using PAb1801. Lane 1: SV80 cells. Lane 2: Normal colonic mucosa. Lane 3: Immunohistochemically positive carcinoma. Lane 4: Immunohistochemically negative carcinoma. Lanes 5 and 6: Immunohistochemically positive carcinomas. Lane 7: SV80 cells. Molecular weights are expressed in kilodaltons.*

villous adenoma that had undergone malignant change, p53 expression was restricted to the frankly carcinomatous portion sparing the dysplastic epithelium. In the carcinomas, the advancing edge was the most consistently staining region. Nonetheless, approximately one half of all colorectal carcinomas did not show p53 expression, and comparison of expressing and nonexpressing carcinomas revealed no differences in terms of the tumor site or established prognostic indicators such as histologic grade,[23] Dukes' stage,[30] and DNA ploidy.[31] Because most of the tumors studied in this consecutive series were (as expected) of Dukes' B and C stages, the lack of correlation with staging is essentially an indicator that p53 expression has no association with the tendency to form lymph node metastasis. It appears, therefore, that p53 expression is associated with the transition from adenoma to carcinoma in some colorectal tumors but does not uniquely determine infiltrative activity or relate to other features of carcinoma progression. However, in view of the short time of clinical follow-up of the patients studied here, we have no data yet that directly compare postoperative course and p53 staining.

The monoclonal antibody PAb1801 used here is specific for human p53, binding near the N terminus.[26] It thus detects both WT and mutated forms of the protein, all the mutations described being 3′ to the antibody binding site.[21] Wild-type p53 has a short half-life and was not detected immunohistochemically in any of the normal tissues in this study. By contrast, mutations of p53, which are common in human colorectal cancer,[21] result in a greatly extended protein half-life permitting immunohistochemical detection.[32] PAb240, which binds selectively to mutated p53,[28] stains positively cases that are PAb1801 positive in this series and has a very similar distribution of reaction product. Thus the tumors exhibiting PAb1801 immunostaining almost certainly have p53 mutations; we are now confirming this by sequencing the gene for p53 in selected tumors (manuscript in preparation).

PAb1801 has the advantage that its epitope is stable in PLPD-fixed (but not formaldehyde-fixed) tissue, thus permitting the improved morphologic localization of paraffin sections. The discrete nuclear localization of the PAb1801 epitope observed here contrasts with a previous report on p53 expression in breast carcinomas based on an analysis of frozen sections in which cytoplasmic staining was observed.[2] Complex formation between mutant p53 and the cytoplasmic heat shock protein hsp 72/73 has been demonstrated, which might explain its cytoplasmic location in some tissues.[14] In a large subset of the present series, however, we also applied PAb1801 to frozen sections and found the same pattern of nuclear staining. Breast carcinomas also differ in that p53-expressing tumors tend to be in poorer prognostic groups,[2] an association we do not observe for colorectal carcinomas (Table 1).

Within some of the sections showing positive immunostaining there was marked cell-to-cell heterogeneity. Because the mutation of the p53 gene probably is clonal, this variation in protein level may indicate that mutated p53 is under similar cell-cycle control to that demonstrated in WT p53 with levels increasing before S phase.[6] Further evidence for cell-cycle–related changes in protein levels comes from the mitotic cells that consistently showed much-reduced immunostaining. The distribution of positively stained cells around the advancing margins of carcinomas has not been reported previously and is

more difficult to explain as a cell-cycle–related phenomenon and may represent some form of inductive interaction with stromal tissues.

In transgenic animals, expression of mutated p53, even at high levels, does not produce tumors in some tissues,[33] indicating that changes in p53 alone are not sufficient to cause neoplastic transformation. In human colorectal carcinogenesis, several other genetic changes occur at high frequency, such as Ki-*ras* mutations and deletions in chromosomes 5q, 18q, and 17p (including the locus of the p53 gene).[34] Accumulation of these events seems to be important in determining prognosis, presumably facilitating the rather poorly defined cellular changes that constitute tumor progression. The data presented here show that expression of p53 (probably due to mutation) is an event occurring around the transition from adenoma to carcinoma and therefore at the same stage in tumor progression as 17p deletion.[34] The distribution of the most intensely staining cells also relates to local infiltrative activity. Therefore p53 may be one factor permitting malignant transformation but it is unlikely to account for other features of carcinoma progression such as metastasis and the tendency to develop DNA aneuploidy.

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